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DNA extraction method with improved efficiency and specificity using DNA methyltransferase and "click" chemistry

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ABSTRACT

In an attempt to develop an alternative method to extract DNA from complex samples with much improved sensitivity and efficiency, here we report a proof-of-concept work for a new DNA extraction method using DNA methyltransferase (Mtase) and "click" chemistry. According to our preliminary data, the method has improved the current methods by (i) employing a DNA-specific enzyme, *Taq*I DNA Mtase, for improved selectivity, and by (ii) capturing the DNA through covalent bond to the functionalized surface, enabling a broad range of treatments yielding the final sample DNA with minimal loss and higher purity such that it will be highly compatible with downstream analyses. By employing Mtase, a highly DNA specific and efficient enzyme, and click chemistry, we demonstrated that as little as 0.1 fg of λ-DNA (close to copy number 1) was captured on silica (Si)-based beads by forming a covalent bond between an azide group on the surface and the propargyl moiety on the DNA. This method holds promise in versatile applications where extraction of minute amounts of DNA plays critical roles such as basic and applied molecular biology research, bioforensic and biosecurity sciences, and state-of-the-art detection methods.

Reliable and highly efficient DNA extraction methods have been a core element of various research fields, including forensics and diagnostics. Tremendous efforts to develop an efficient method to extract, and thereby isolate, DNA with high sensitivity and selectivity from complex samples in a time-saving manner have met with success. For example, silica (Si)[1]-based resins or membranes integrated into a microchip for high-throughput DNA analysis have been particularly successful and are commonly used [1]. However, as DNA has become an important player in providing pivotal clues in research, forensics, and diagnostics, requirements of successful DNA extraction methods have been redefined, as have those for subsequent analyses. Therefore, conventional methods for DNA extraction from complex samples, which mostly include a multistep process (cell lysis, breakdown of DNA-related proteins, and then isolation of DNA using various chemistries such as ethanol precipitation and selective binding of DNA to Si-based surface or glass fiber matrix), have been challenged due to lower compatibility with downstream analyses [2]. That is, the majority of the commercially available methods are based on "boom" chemistry [1], which relies on binding between nucleic acids and Si in the presence of high concentrations of chaotropes such as 6 to 8 M guanidine or detergent. Although these approaches prove to be sufficient for some applications, they also suffer several shortcomings. First, due to the nonspecific separation mechanism based on ionic interaction, they yield "not so perfectly pure" final samples contaminated with other negatively charged polymers such as polysaccharides, which further complicates the downstream analyses. Second, they require high concentrations of salt as a chaotrope to disrupt the biomembranes of the sample and mix up DNAs of different biological origins. Third, current commercial methods are designed primarily to treat samples of smaller volumes (μl to ml) to accommodate high-throughput procedures.

Ideally, a new DNA extraction method should combine improved purity and higher yields with better compatibility with downstream analyses. The new technique should be highly selective for the DNA of interest in a complex sample and able to extract DNA in a noninvasive manner. In addition, it should be highly adaptable to a broad range of downstream analyses and to high-throughput systems, requiring effectiveness over a range of volumes from a few microliters to several liters.

Among DNA-specific enzymes, DNA methyltransferases (Mtases) catalyze the transfer of methyl groups to DNA from a cofactor,

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[1] *Abbreviations used:* Si, silica; Mtase, methyltransferase; SAM, *S*-adenosyl-L-methionine; MTaqI, *Taq*I Mtase; CH_2Cl_2, dichloromethane; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; ES–MS, electrospray mass spectroscopy; RTaqI, *Taq*I endonuclease; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; 2-butynyl–SAM, 2-butynyl-modified SAM; BSA, bovine serum albumin.

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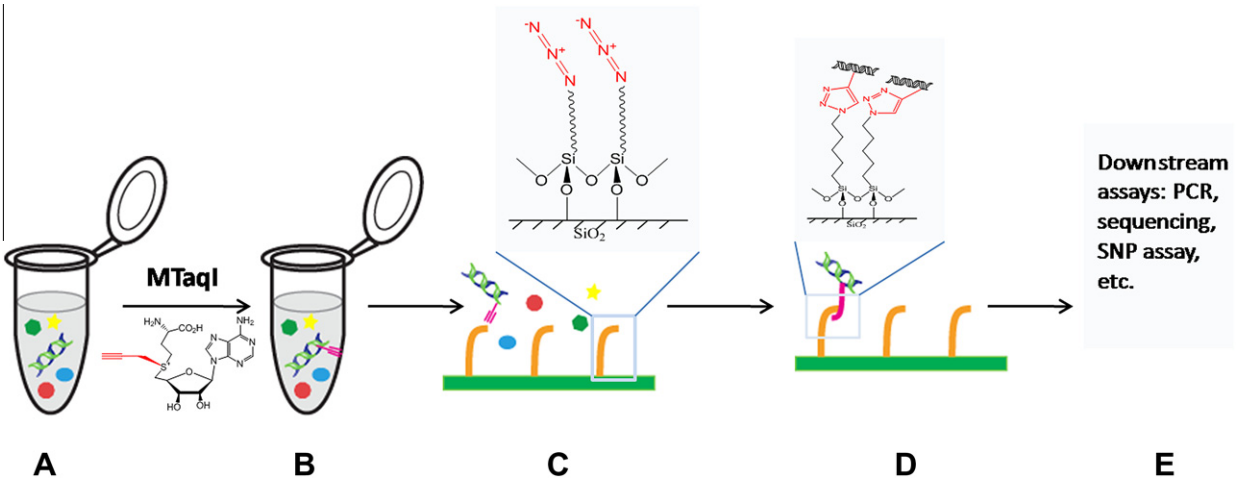


Scheme 1. Schematic view of the assay method. DNA in the complex sample (A) will be selectively labeled (B) using MTaqI and alkynyl–SAM. The crude mixture from step B will be applied to azide-activated surface in the presence of Cu^{2+} (C). The other components will be washed away, whereas the DNA will be covalently attached to the surface (D), which can be used for myriads of downstream analyses (E).

S-adenosyl-L-methionine (SAM), on recognition of short nucleotide sequences (4–7 nt). The mechanism of DNA Mtase and exploitation of the enzyme for numerous pharmaceutical purposes have been of particular interest [3]. Most recently, chemically modified SAM was suggested to provide a chemical probing tool for DNA Mtase-related biochemical reactions [4]. Dalhoff et al. demonstrated that the *Taq*I Mtase (MTaqI) from *Thermus aquaticus* uses a "propargyl group" to modify the DNA instead of the natural methyl moiety from SAM [4].

Since its introduction by Sharpless and coworkers in 2001, "click" chemistry has received tremendous attention due to its ability to irreversibly couple two molecular modules under mild conditions [5]. A typical example of click chemistry is 1,3-dipolar cycloaddition between a propargyl group and an azide moiety, This particular click chemistry has been widely used for biological applications because both moieties are easily introduced into biomolecules and are stable under physiological conditions. Several groups have reported efficient immobilization of propargyl-carrying synthetic oligonucleotides onto the azide-modified surfaces with and without a Cu(II) catalyst [6,7]. In those studies, chemically assembled short oligonucleotides were modified with propargyl groups on their bases before application to various azide-activated surfaces such as gold, silica, and biodegradable polymer fibers [8]. Altogether, these studies clearly demonstrate the versatility of the matrices for the azide-activated surfaces and a broad range of application formats.

Therefore, inspired by the previous studies, we employed DNA Mtase and a propargyl analogue of the cofactor SAM to modify DNA molecules and further immobilize them on the azide-modified surface through covalent bonding. Scheme 1 demonstrates a brief summary of the designed assay method. After extensive washing, this method yields desired DNA with greater purity without contaminating inorganic and organic species. The high specificity of DNA Mtase toward DNA and the covalent bond between the surface and the DNA enable the protocol to be highly selective and efficient.

Materials and methods

The chemicals and organic solvents, such as dichloromethane (CH_2Cl_2) and dimethylformamide (DMF), were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless indicated otherwise. SAM was chemically modified and purified using C18 high-performance liquid chromatography (HPLC) as a diastereo mixture and characterized using electrospray mass spectroscopy (ES–MS) [4]. The oligonucleotides used as standard substrates were purchased from Integrated DNA Technology (IDT, Coralville, IA, USA). The Si beads were purchased from Bangs Laboratory (Fishers, IN, USA). MTaqI, *Taq*I endonuclease (RTaqI), and Dam(−) λ-DNA were purchased from New England Biolabs (Ipswich, MA, USA). MTaqI was also overexpressed as the poly-His form in an *Escherichia coli* system and purified using standard molecular biology and biochemistry techniques. Polymerase chain reaction (PCR)-related products were purchased from Invitrogen (Carlsbad, CA, USA). The beads were rinsed with phosphate-buffered saline (PBS) by repeating spin down and resuspension multiple times (three times in most cases). When spinning, the spinning speed did not exceed 150 rcf to prevent clumping of the beads.

Synthesis and purification of 2-butynyl–SAM

The synthesis of 2-butynyl-modified SAM (2-butynyl–SAM) was performed following the procedure described previously [4]. Briefly, poly(4-vinylpyridine) (2.52 g, 24.0 mmol of pyridine residues) was added to dry CH_2Cl_2 (15 ml) before cooling the suspension to 0 °C with an ice bath and the addition of trifluoromethanesulfonic anhydride (6.21 g, 22.0 mmol) under argon gas. Solution of 2-butynyl-1-ol (1.5 ml, 20 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise using an airtight syringe to this stirred suspension under argon gas within 20 min. After stirring at room temperature for 10 min, poly(4-vinylpyridine) was removed by filtering through a glass-bottom flask and rinsed with CH_2Cl_2 to further extract the product, and the pooled organic layer was concentrated using a rotary evaporator. The solution of *S*-adenosyl-L-homocysteine (20 mg, 52 μmol) in a 1:1 mixture of formic acid and acetic acid (1 ml) was cooled in an ice bath before the addition of the activated butynyl moiety from above. The reaction was quenched after 2 h by adding water (20 ml). The organic side products were removed by extracting with diethylether (10 ml) three times, and the aqueous fraction was freeze-dried overnight. The next day, the dried sample was dissolved in 3 ml of water and purified using a C18 reverse phase HPLC system eluting with methanol (linear gradient 0 to 100% in 15 min) in aqueous ammonium formate (20 mM, pH 3.5) by monitoring at 260 nm. The purified fractions were pooled, freeze-dried, and characterized by ES–MS (M^+ = 423 for propargyl–AdoMet, M^+ = 437 for 2-butynyl–AdoMet).

Preparation of azide-modified Si beads

The Si beads (500 μl suspension, 2 μm, Bangs Laboratory), previously rinsed with ethanol (95%, 1 ml), water (1 ml), and CH_2Cl_2 (1 ml) by repeated spin-down (~110 rcf, 3 min) and resuspension, were resuspended in 1 ml of 1% (v/v) 1-bromoundecyltrichlorosilane in CH_2Cl_2. After incubation with gentle agitation at room temperature for 1 h, the suspension was rinsed with CH_2Cl_2, ethanol, and DMF. The beads then were incubated in 1 ml of saturated NaN_3 solution in DMF at room temperature overnight, followed by rinsing with DMF, ethanol, and water (2 ml each, repeated three times).

MTaqI reaction

λ-DNA (5 ng/μl), Triton X-100 (0.01%), bovine serum albumin (BSA, 0.1 mg/ml), 2-butynyl–SAM (50 μM), and MTaqI (0.5 U) were mixed in a total volume of 40 μl in NEB buffer 4 (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9) before incubation at 65 °C for 2 h. The reaction was quenched by heating the mixture at 80 °C for 20 min.

Restriction protection assay using RTaqI

After the Mtase reaction, 1 μl of RTaqI (New England Biolabs) was added to an aliquot (20 μl) of the Mtase reaction mixture and incubated at 60 °C for 30 min. The protection of the corresponding DNA by Mtase in the presence of natural and chemically modified cofactor SAM was monitored using 0.8% agarose gel.

"Click" chemistry between alkyne-modified DNA and azide-activated beads

The Mtase reaction mixture from above (total 40 μl) was mixed with 20 μl of azide-modified bead suspension in 100 μl of ascorbate solution (containing 1 mg of sodium ascorbate and 0.5 mg of disodium bathophenanthroline disulfonate in 5 ml of water) at room temperature overnight. The next day, the beads were rinsed with H_2O (1 ml each, repeated three times) and ethanol (1 ml each, repeated three times) before resuspension in 10 μl of PBS.

"On-bead" PCR

The suspension above (2 μl) was used as the template in a PCR reaction by *Taq* polymerase (95 °C for 4 min, denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1.5 min, 35 cycles from second step to fourth step, final elongation at 72 °C for 10 min, and then keep at 4 °C). The PCR product was identified on 0.8% agarose gel and sequenced to identify the amplified sites by the polymerase.

Mtase reaction in a broad range of pHs

λ-DNA (5 ng/μl), Triton X-100 (0.01%), BSA (0.1 mg/ml), 2-butynyl–SAM (50 μM), and MTaqI (0.5 U) were mixed in a total volume of 40 μl in a buffer (prepared to be in pH range of 2.0–11.0, as directed at http://www.delloyd.50megs.com/moreinfo/buffers2.html) before incubation at 65 °C for 2 h. The reaction was quenched by heating the mixture at 80 °C for 20 min.

Mtase reaction in a broad range of common salts

Mtase reaction was carried out as described above (λ-DNA (5 ng/μl), Triton X-100 (0.01%), BSA (0.1 mg/ml), and 2-butynyl–SAM (50 μM) were mixed in a total volume of 40 μl in NEB buffer 4 (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9) at 65 °C for 2 h) in the presence of common salt over a range of concentrations. The final pH after the addition of each salt was monitored before the addition of MTaqI (0.5 U).

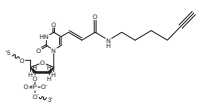
Standard DNA extraction from soil samples without cell membrane disruption

The garden soil outside our laboratory (100 mg/sample), 1 ml of buffer (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9), and standard substrate (λ-DNA) of varying concentrations were mixed in a plastic tube at room temperature. The soil samples and buffer were centrifuged (2000*g* for 3 min), and the supernatant was collected (repeated three times). The pooled supernatant was centrifuged (2000*g* for 5 min, repeated three times) to further remove the concomitant debris before subjecting the final supernatant to the Mtase reaction followed by click chemistry with the azide-activated beads (2 μm) as described above.

DNA extraction from model microbial sample

A bacterial sample was artificially prepared using *E. coli* (ATCC 4157). First, *E. coli* was grown to OP_{600} = 0.2 in Luria–Bertani (LB) medium before being spun down to a pellet. The pellet was then rinsed with PBS three times before storage at −80 °C. The frozen pellet was mixed with 1 ml of extraction buffer (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9), rapidly frozen by immersion in liquid nitrogen (2 min), and then thawed at 60 °C (5 min); this freeze–thaw cycle was carried out three times. Each tube containing the resulting solution was shaken at high speed for 2 min in a bead mill homogenizing unit (BioSpec Mini-Bead Beater). The selection of bead size and the proportion of beads to cell suspension were determined by following guidelines for disrupting bacterial cells provided by the manufacturer. After removing the beads and solid debris from the lysate by centrifugation, aliquots of different volume from the supernatant (50, 100, 300, and 500 μl) were

Fig. 1. Sequences of standard oligonucleotide substrates.

Substrate 1: 5'-ggatccgaattcgcgctcgatcgcgcggatccgaattcgcgc**T**cgatcgcgc-Fluorescein-3', **T**= 

Substrate 2:

5'-6FAM-ttaattaaTCGAattgtaatacgactcactatagggagaggatccgaattcgcgctagatcgcg ctagcgcggccgcgctgagcaataa-3'

(6-FAM = 6-carboxy-Fluorescein)

Fig.1. Standard oligonucleotide substrates.

transferred to separate plastic tubes. Mtase reaction followed by click chemistry with the azide-activated beads was carried out as described above.

Results and discussion

The purpose of this article was to report a successful study on proof of concept to use DNA Mtase and "click" chemistry to further develop as a better alternative to the current DNA extraction methods. To prove that this new platform will provide a new venue for the research areas where minute amounts of pure DNA play important roles, we first established a general protocol for the Mtase method and then investigated the method with the samples in "not that simple" conditions. To establish the general protocol, the planar and spherical Si surfaces were first chemically modified as described previously [9]. Successful modification of the surface with an azide (N_3) group was confirmed by adding the propargyl–amine and subsequent fluorescent labeling (see Supplementary Fig. 1 in supplementary material). To confirm that click chemistry is functional with DNA molecules, we then designed two standard oligonucleotides (100 nt), each with a fluorescent probe: one with a nucleotide bearing a propargyl-modified base (Fig. 1, substrate 1) and the other with MTaqI recognition site TCGA (Fig. 1, substrate 2). After the click chemistry procedure, standard substrate 1 was successfully isolated, as indicated by fluorescently labeled Si beads. Then substrate 2 was treated with MTaqI in the presence of 2-butynyl–SAM [4] at 65 °C for 2 h before incubation with $CuSO_4$ in ascorbic acid solution (1 mM) [5]. Images from a fluorescence microscope show that substrate 2 was successfully bound to the azide-activated beads only in the presence of both MTaqI and 2-butynyl–SAM (Fig. 2), whereas the sample with natural cofactor SAM did not yield fluorescing beads, proving that only the oligonucleotides modified with 2-butynyl–SAM were associated with the beads. The restriction protection assay (see Supplementary Fig. 2 in supplementary material) was used to investigate MTaqI efficiency with a series of chemically modified

Fig.4. Study on limit of detection. The Mtase method yielded PCR product of the right size and sequence using DNA as low as 0.1 fg as a template (lane 1: beads only without DNA; lane 2: supernatant from final wash; lanes 3–14: different concentrations of DNA (0.1 fg (lane 3), 1 fg (lane 4), 10 fg (lane 5), 100 fg (lane 6), 1 pg (lane 7), 10 pg (lane 8), 100 pg (lane 9), 500 pg (lane 10), 1 ng (lane 11), 10 ng (lane 12), 100 ng (lane 13), and 500 ng (lane 14); lane 15: soluble-free DNA template).

cofactors, including 2-butynyl–SAM, propargyl–SAM, and the natural cofactor SAM, where 2-butynyl–SAM showed as good efficiency as the natural cofactor SAM.

Once it was confirmed that the enzymatically modified DNA was successfully bound to the azide-activated Si surface, we used the beads as a template for "on-bead" PCR. Using Dam(−) λ-DNA (linear DNA with 48,502 bp contains 121 recognition sites for MTaqI) (New England Biolabs), we performed the MTaqI reaction followed by click chemistry with azide-modified Si beads. Each supernatant from the washing steps was subjected to PCR to detect the presence of unbound DNA. Further quantification after click chemistry using a spectrophotometer suggested that immobilization was approximately 90% complete (<10% loss of sample DNA). This result demonstrates that the Mtase method provides an alternative to the current methods with much improved yield over current membrane-based methods, a common drawback of which is low yield due to nonspecific binding. In addition, PCR products of the right sizes and sequences were obtained from reactions of the 2-butynyl–SAM-modified λ-DNA with selected polymerases, including *Taq*, Deep Vent, and *Pfu* DNA polymerase (Fig. 3).

Due to the high specificity between DNA and Mtase, this method is expected to capture DNA as small as 15 bp. Therefore, after determining the yield of the on-bead PCR reactions, we investigated the sensitivity of the method compared with current commercial kits. The serially diluted standard DNA (10 ng to 0.1 fg of λ-DNA) was treated with MTaqI and 2-butynyl–SAM at

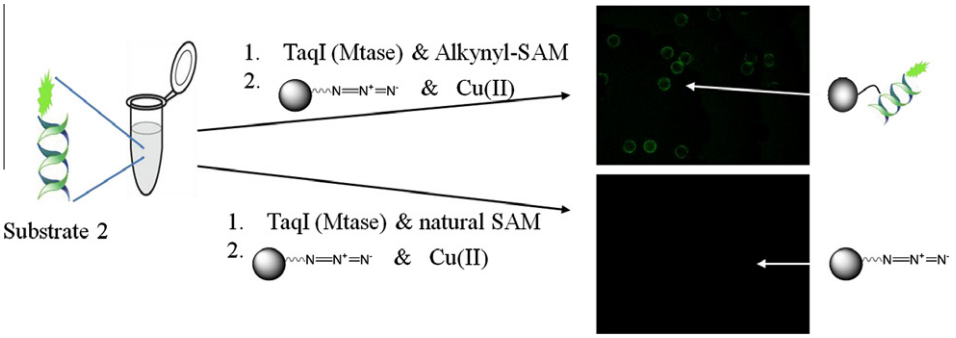


Fig.2. MTaqI reaction with alkynyl–SAM (top image) or natural SAM (bottom image) followed by click chemistry with Cu^{2+}.

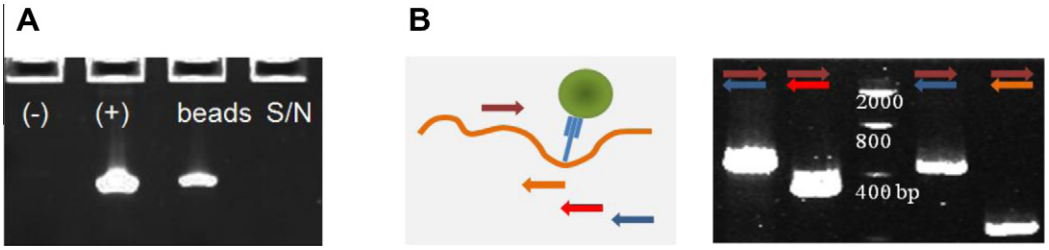


Fig.3. The modified site is tolerated by polymerase. (A) PCR product from the beads with no DNA (−), from the soluble free template (+), from the beads with DNA after click chemistry (beads), and from the supernatant after final wash (S/N). (B) Alkyne labeled DNA-bound bead showing a series of primers (left) and the PCR products from the primers with alkynyl modification (right) in various sites within and outside amplification region (first left lane: (+) control without alkynyl modification of DNA).

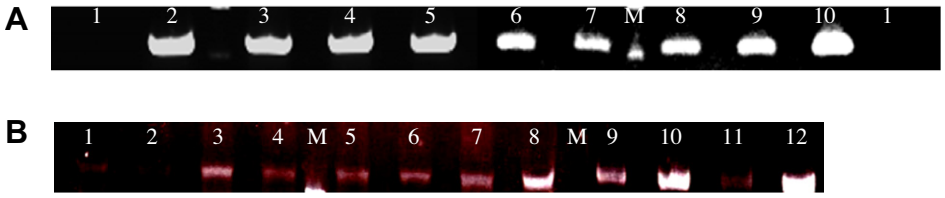


Fig.5. Efficiency of Mtase method in a broad range of pHs (A) and in the presence of common salts (B). (A) PCR products using the beads from different pHs (lane 1: pH 2.0; lane 2: pH 3.0; marker; lane 3: pH 4.0; lane 4: pH 5.0; lane 5: pH 6.0; lane 6: pH 7.0; lane 7: pH 8.0; marker; lane 8: pH 9.0; lane 9: pH 10.0; lane 10: (+) control, free λ-DNA; lane 11: (−) control, beads without DNA). (B) PCR products using the beads with DNA extracted from high salt concentrations (lane 1: 10 mM $CaCl_2$; lane 2: 1 mM $CaCl_2$; lane 3: 100 nM $CaCl_2$; lane 4: 10 nM $CaCl_2$; marker; lane 5: 10 mM NaCl; lane 6: 1 mM NaCl; lane 7: 100 nM NaCl; lane 8: 10 nM NaCl; marker; lane 9: 10 mM $MgCl_2$; lane 10: 1 mM $MgCl_2$; lane 11: 100 nM $MgCl_2$; lane 12: 10 nM $MgCl_2$).

Fig.6. PCR products using the DNA-bound beads after Mtase click chemistry. (A) PCR products from the soil samples with 50 pg of λ-DNA (lane 1), 500 pg of λ-DNA (lane 2), 5 ng of λ-DNA (lane 3), 50 ng of λ-DNA (lane 4), marker, and (+) control with free λ-DNA (lane 5). (B) PCR products using (+) control, purified DNA from *E. coli* (ATCC 4157) (lane 1); (+) control, the supernatant (1 ml) after lysis (lane 2); the following *E. coli* suspension samples after Mtase click purification: 50 μl (lane 3), 150 μl (lane 4), 300 μl (lane 5), 500 μl (lane 6), 700 μl (lane 7), and 1 ml (lane 8); and (−) sample without *E. coli* (lane 9).

60 °C as described in Materials and Methods. Following the click chemistry procedure, the beads with DNA were rinsed before performing PCR on an aliquot of bead suspension to measure the DNA detection limit. The Mtase method yielded PCR product from all beads of different DNA concentrations as low as 0.1 fg (Fig. 4), quantification of which was further confirmed using an Agilent BioAnalyzer (Santa Clara, CA, USA). Our results show that, compared with the currently available DNA extraction kits (most of which show sensitivity in terms of nanograms of DNA), the Mtase method enables detection of DNA with single-digit copy number. Another requirement of a successful new assay should be robustness of the procedure in various sample conditions. Therefore, we investigated efficiency of the Mtase method in a broad range of pH and salt concentrations. The PCR product after Mtase and click chemistry showed that the Mtase method is effective in the range of pH 4.0 to pH 10.0 and in the presence of nanomolar (nM) to millimolar (mM) concentrations of common salts such as Ca^{2+}, Mg^{2+}, and Na^{+} (Fig. 5).

The preliminary data above clearly suggest that the Mtase method is a better alternative with improved sensitivity, so we tested its ability to capture DNA of interest with high selectivity from a complex sample. To determine its efficiency, we evaluated the Mtase method with samples of DNA mixed with soil and *E. coli* lysate. First, the soil samples were prepared with the known amount of the standard λ-DNA to demonstrate that the free-floating DNA in complex samples is selectively extracted despite the presence of concomitant components such as large particles, metals, and other biopolymers (e.g., proteins contained in soil). As shown in Fig. 6A, the free exogenous DNA in the sample was successfully extracted and subjected to PCR, giving the amplified product of the right size and sequence. Second, we extracted endogenous DNA from a cultured *E. coli* sample where the bacterial membrane was disrupted by free thaw and bead beating. We confirmed that the MTase method functions effectively, as indicated by PCR products of the right size and sequence (Fig. 6B).

Extraction of DNA from complex samples plays a pivotal role in diverse research fields. This possible alternative that demonstrates improved sensitivity and efficiency can be exploited for broader applications compared with the current methods. This will open new opportunities for studying complex or low copy number samples, for which current methods might not be as efficient as desired. Unlike the current methods that use either filtration or generation of a paramagnetic field, the use of DNA Mtase and subsequent click chemistry offers advantages with regard to substrate specificity and broader compatibility to downstream analyses. Therefore, the success of this proof-of-concept study for the Mtase method reported here holds promise in many different aspects.

Currently, development of reliable new methods to extract DNA under various conditions is urgently needed in many fields of research due to limitations of commonly used methods. These limitations include relatively poor yield of DNA recovery and limited sample volumes from membrane-based filtration systems (PureYield Midiprep and MagneSil by Promega) and/or paramagnetic particle-based separation systems (Maxwell 16 by Promega and Agencourt DNAdvance by Beckman Coulter Genomics). However, the Mtase method provides a new platform for DNA extraction to address these challenges. First, the Mtase method can be applied to a broad range of sample volumes by increasing the concentration of Mtase and azide-activated surface, thereby offering a more efficient alternative for samples of low biomass in larger volumes. Second, the Mtase method demonstrated high sensitivity and selectivity for DNA as low as a femtogram of DNA, which is close to a thousand-fold increase in sensitivity compared with the current common methods. Third, the Mtase method can be combined with a broader range of downstream analyses due to purity of the sample. Further optimization of the protocol for different samples types and development of various surface formats, such as magnetic beads and incorporation into the plastic tube surface, will increase the range of applications and utility of this assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.03.017.

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